PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
G01N 33/50, 33/68, 33/564	A1	WO 99/34209
		(43) International Publication Date: 8 July 1999 (08.07.99)

(21) International Application Number: PCT/US98/26868

(22) International Filing Date: 17 December 1998 (17.12.98)

(30) Priority Data:
60/070,298 31 December 1997 (31.12.97) US

(71) Applicant: THE BRIGHAM AND WOMEN'S HOSPITAL, INC. [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventors: HAFLER, David, A.; 110 Forest Avenue, West Newton, MA 02165 (US). STROMINGER, Jack, L.; 2030 Massachusetts Avenue, Lexington, MA 02173 (US). WILSON, Brian; 33 Homestead Street, Lexington, MA 02173 (US). KENT, Sally, C.; 28 Sidlaw Road #9, Brighton, MA 02135 (US).

(74) Agent: SANZO, Michael, A.; Vinson & Elkins L.L.P., 2300 First City Tower, 1001 Fannin, Houston, TX 77002-6760 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DIAGNOSTIC AND THERAPEUTIC METHODS BASED UPON Vα24JαQ T CELLS

(57) Abstract

The present invention is directed to methods that can be used for diagnosing whether an individual either has, or is likely to develop, an autoimmune disease. The methods are based upon determining the level of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells present in the individual being tested or the pattern of cytokine secretion evidenced by these cells. In addition, the invention is directed to a therapeutic method for treating or preventing autoimmune disease which is based upon the specific expansion of the $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cell population.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Diagnostic and Therapeutic Methods Based Upon Vα24JαQ T Cells

Field of the Invention

The present invention is directed to methods for diagnosing and treating autoimmune diseases. Diagnostic methods are based upon the discovery that $CD4^-CD8^-$ Vα24JαQ$^+$ T cells are lost in response to the onset of disease and also develop an altered pattern of cytokine secretion. Therapeutic methods are based upon the discovery of a means for selectively expanding this subset of T cells.

Background of the Invention

Autoimmune diseases are the result of a patient's immune system attacking their own cells and tissues. This can result in a wide variety of diseases, including multiple sclerosis, myasthenia gravis, rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus, psoriasis, scleroderma, idiopathic thrombocytopenia purpura, and Sjögen's disease. For the most part, the etiology of autoimmune diseases is poorly understood, and attempts at therapeutic intervention have met with limited success.

Recently, it has been discovered that T lymphocytes exist in subpopulations characterized by different patterns of cytokine secretion (Abbas *et al.*, *Nature 383*:787–793 (1996)). The Th1 subset of $CD4^+$ T cells promotes inflammatory cellular immune responses and is biased toward the secretion of IFN-γ, TNF-β, and IL-2. Th2 cells are biased towards the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13, induce humoral immunity, and inhibit Th1 responses. In certain autoimmune diseases, *e.g.*, in type 1 diabetes, it appears that the Th1 pattern of secretion often becomes predominant but the cellular mechanisms integrating the drive to Th1 or Th2 are poorly understood (Kallmann *et al.*, *Diabetes 46*:237–243 (1997)). One possibility is that disease onset is associated with an expansion or loss of groups of T cells with particular secretory characteristics.

In the mouse, a bias toward Th2 cells may be promoted by the activation of T cells having the Vα14Jα281 receptor (Bendelac *et al.*, *Ann. Rev. Immunol. 15*:535–562 (1997); Vicari *et al.*, *Immunol. Today 17*:71–76 (1996)). Humans have been shown to have a population of T cells expressing a receptor, Vα24JαQ, with a close sequence homology to Vα14Jα281 (Porcelli *et*

al., J. Exp. Med. 178:1–16 (1993)). Defining the relationship between changes in these cells and the onset of autoimmune diseases may lead to new diagnostic and therapeutic procedures.

Summary of the Invention

The present invention is based upon the discovery that there is a subset of T cells that decreases in number and which undergoes a change in its cytokine secretion pattern as humans develop autoimmune disease. Cell loss and changes in cytokine secretion become more pronounced as individuals progress from a normal to a diseased state. In addition, therapeutic procedures have been developed to prevent T cell loss and that can be used to prevent or treat autoimmune diseases.

In its first aspect, the invention is directed to a method for evaluating the likelihood that a human subject either has, or will develop, an autoimmune disease by determining the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells in their circulation. This number is compared with the number of such cells present in a control group comprised of individuals known to be free of autoimmune disease and the difference between the values obtained for the tested subject and control group is then correlated with the likelihood of the subject either having or developing autoimmunity. Likelihood increases as the number of $V\alpha24J\alpha Q^+$ cells in the tested subject decreases relative to the number of such cells present in the control group. Among the autoimmune diseases that may be tested for using this procedure are multiple sclerosis; systemic lupus erythematosus; rheumatoid arthritis; type 1 diabetes; myasthenia gravis; psoriasis; scleroderma; Sjögen's disease; and idiopathic thrombocytopenia purpura.

One variation of the diagnostic method described above involves the determination of the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ in the subject being tested and comparing this to the percentage of such cells in a control group of disease-free individuals. Again, the likelihood of the tested individual having or developing an autoimmune disease increases as the percentage of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells in this individual decreases relative to the percentage of these cells in the control group. One way that percentages may be determined is by sorting all $CD4^-CD8^-$ $\alpha\beta$ TCR^+ T cells using flow cytometry; amplifying $V\alpha24$ transcripts and sequencing the TCR CDR3 region of the amplified product to determine frequency; and then multiplying this frequency by the percentage of total T cells that are

$CD4^-CD8^-$ $V\alpha24^+$. This procedure may be applied to the autoimmune diseases listed above and, in particular, to type 1 diabetes.

In another aspect, the present invention is directed to a method for determining the likelihood that a human subject will develop an autoimmune disease based upon the types of cytokines secreted by $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells. This method is based upon the discovery that, when isolated from normal individuals, these cells secrete substantial levels of both interferon gamma (IFN-γ) and interleukin-4 (IL-4), whereas cells from patients with autoimmune disease preferentially secrete IFN-γ. Thus, the method involves isolating $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells from the test subject, determining the extent to which these cells secrete IL-4, and then correlating secretion with the likelihood of the subject having or developing an autoimmune disease. Likelihood decreases as the secretion of IL-4 increases. Preferably, the determination of IL-4 secretion should be performed after the isolated T cells are stimulated with a T cell activating factor such as anti-CD3 antibody. Secretion levels of IL-4 in cells derived from normal individuals can be used as a basis for comparison.

The method can also be performed by determining the ratio of IL-4 to IFN-γ secretion in the isolated cells. In this case, the likelihood of an individual having or developing an autoimmune disease decreases as the ratio increases, *i.e.*, as the levels of IL-4 rise with respect to the levels of IFN-γ. Again, the secretion ratio in cells derived from normal individuals can be used as a basis of comparison. The method can be used for detecting the autoimmune diseases discussed above and, in particular, for detecting individuals that have or are likely to develop type 1 diabetes.

In another aspect, the present invention is directed to a method of treating a human subject that has or is likely to develop an autoimmune disease by increasing the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells in their circulation, preferably by exposing the cells to CD1d or a digestion product of CD1d. Clonal expansion may take place after T cells are removed from an individual, or, alternatively, the T cell subpopulation may be expanded *in vivo*. The therapeutic procedure may be used for multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, myasthenia gravis, psoriasis, scleroderma, Sjögen's disease, or idiopathic thrombocytopenia purpura.

Detailed Description of the Invention

The present invention is based upon the concept that there are certain subsets of T cells that can contribute to the development of autoimmune diseases. This concept is supported by experiments performed on patients with type 1 diabetes and their non-diabetic identical siblings. The results indicate that the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ is substantially reduced in diabetics relative to their "at-risk" non-diabetic siblings. In addition, it was found that $CD4^-CD8^-$ $V\alpha24J\alpha Q$ cells cloned from the diabetics evidenced a significantly different pattern of cytokine secretion in response to stimulation with anti-CD3 antibody. Specifically, these clones were found to secrete only IFN-γ, whereas nearly all clones derived from non-diabetic siblings secrete both IL-4 and IFN-γ.

Other results indicated that the specific subset of T cells associated with the onset of autoimmune disease, *i.e.*, the $CD4^-CD8^-$ $V\alpha24J\alpha Q$ cells, proliferate rapidly when exposed to CD1d. This provides a means for expanding this T cell subset in individuals with abnormally low levels such as those that have or are at risk for developing autoimmunity.

A. Diagnostic Methods for Detecting Autoimmunity Based on the Number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T Cells Present in an Individual

There are two basic methods that may be used for determining whether a particular individual either has, or is likely to develop, an autoimmune disease. The first is based upon the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells that the individual has relative to the number found in a population of normal individuals, *i.e.*, individuals that do not have autoimmune disease. One method that has been demonstrated to be effective for quantitating the T cell subset is to separate $CD4^-CD8^-$ αβ T cell receptor-containing cells by flow cytometry and to then amplify all Vα24 transcripts by PCR. The amplification product is sequenced to determine the frequency of $V\alpha24J\alpha Q^+$ sequences, and this frequency is then multiplied by the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24^+$ as determined by flow cytometry. The product equals the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$.

The percentage determined in the manner described above can be compared directly with similarly determined percentages in a normal population or the percentages can be readily converted into cell numbers and compared. In either case, there is a direct correlation between

a decrease in this subset of T cells and either the likelihood that the subject being tested will develop an autoimmune disease or that they already have such a disease that has not yet progressed to the point where overt clinical manifestations are present. Of course, the same assay can be used to confirm a diagnosis of autoimmune disease made by other criteria.

Although the procedure described above is effective for determining whether there has been a decrease in an individual's T cells, the invention is compatible with other procedures as well. For example, antibodies specifically directed to a cell surface antigen exclusively present on Vα24JαQ T cells may be used in standard immunoassays for quantitating cell number. Methods for making and selecting such antibodies are well known to those of skill in the art, as evidenced by standard reference works such as: Harlow *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988); Klein, *Immunology: The Science of Self-Non-Self Discrimination* (1982); and Kennett *et al.*, Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses (1980). Once produced, antibodies can be used in radioimmunoassays or immunometric assays, also known as "two site" or "sandwich" assays (*see* Chard, "An Introduction to Radioimmune Assay and Related Techniques," in Laboratory Techniques in Biochemistry and Molecular Biology, North Holland Publishing Co., N.Y. (1978)). In a typical immunometric assay, a quantity of unlabeled antibody is bound to a solid support that insoluble in the fluid being tested, *e.g.*, blood, lymph, cellular extracts, etc. After the initial binding of antigen to immobilize antibody, a quantity of detectably labeled second antibody (which may or may not be the same as the first) is added to permit detection and/or quantitation of bound antigen (*see, e.g.*, Radioimmune Assay Method, Kirkham *et al.*, ed., pp. 199–206, E & Livingstone, Edinburogh (1970)). Many variations of these types of assays are known in the art and could be used to quantitate Vα24JαQ$^+$ cells. Binding assays could also be performed using a labeled ligand that binds specifically to the Vα24JαQ$^+$ cells. As examined further in the Examples section, labeled CD1d protein, or fragments of this protein, should be suitable for this purpose.

B. Diagnostic Methods for Detecting Autoimmunity Based on the Cytokine Secretion Pattern of CD4$^-$CD8$^-$ Vα24JαQ$^+$ T Cells

An alternative method for determining whether an individual has, or is likely to develop, an autoimmune disease is based upon the cytokines secreted by CD4$^-$CD8$^-$ Vα24JαQ$^+$ T cells.

It has been experimentally demonstrated that the onset of autoimmune disease is associated with a decrease in IL-4 secretion by these cells relative to secretion seen in cells derived from normal individuals. Secretion of IFN-γ by the cells is relatively unchanged. Thus, a determination may be made either of IL-4 secretion *per se*, or the ratio of IL-4 secretion relative to IFN-γ secretion. In either case, best results are obtained when cells are stimulated with a factor such as anti-CD3 antibody.

The first step in the procedure requires that the relevant cells be sorted using flow cytometry and commercially available antibodies and that the sorted cells be grown to determine if they have the Vα24JαQ form of receptor. The identified clones may then be stimulated with commercially available anti-CD3 antibody and supernatants, then assayed for IL-4 concentration and, if desired, IFN-γ concentration. In each case, this may be accomplished using ELISA assays. As discussed in the Examples section, there is a close correlation between a bias toward IFN-γ secretion by the cells and autoimmune disease, particularly type 1 diabetes.

C. Expansion of Vα24JαQ Cells as a Therapy

It has been demonstrated that $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells can be clonally expanded by exposing the cells to CD1d. Thus, individuals that are identified as having abnormally reduced levels of these cells and therefore being at risk for the development of an autoimmune disease, may be treated using either this protein or peptides derived from the protein. For example, T cells may be isolated from a patient, exposed to transfectants expressing CD1d to increase the proportion of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells, and then reintroduced into the patient.

An alternative, and generally preferable method, is to treat an individual directly by injecting CD1d or a T-cell activating fragment derived from this protein. The exact dosage to be given to a patient will be determined using standard clinical techniques and the effect of administration will be followed using periodic assays to determine whether $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells have increased in number. Once cells have reached a level typical of that present in normal individuals, administration should cease and the patient should be monitored to determine if levels again fall. If this occurs, more therapeutic agent may be administered.

Any route of administration and dosage form is compatible with the present invention and T cell stimulatory agent may be administered either as the sole active agent or in combination with other therapeutically active drugs. In general, non-oral routes of administration are preferred in order to avoid the degradation of CD1d. All dosage forms, whether tablets, pills, capsules, powders, aerosols, suppositories, skin patches, or parenterals, may be prepared using methods that are standard in the art (*see, e.g.*, Remington's Pharmaceutical Sciences, 16th ed., A. Oslo, editor, Easton, P.A. (1980)) and dosage forms may be prepared in conjunction with any of the vehicles and excipients commonly employed in pharmaceutical preparations, *e.g.*, talc, gum arabic, lactose, starch, magnesium sterate, cocoa butter, aqueous or non-aqueous solvents, oils, paraffin derivatives, glycols, etc. Coloring and flavoring agents may also be added to preparations, particularly to those for oral administration.

D. Utility

The present invention provides a means for determining the likelihood that an individual will develop an autoimmune disease such as type 1 diabetes or multiple sclerosis. The test can be used for patients with a family history of autoimmune disease or it can be used for screening the population at large. Once an individual is identified as having abnormally low levels of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells, they can be monitored more closely and administered agents designed to treat or prevent the progression of autoimmunity. The results reported herein suggest that the diagnostic methods are highly reliable as a predictor of disease progression and onset, and that specific expansion of cells as a therapy can be achieved using CD1d.

Examples

I. Materials and Methods

Antibodies and phenotypic analyses of T cells. Flow cytometry experiments were performed on FACScaliber and FACS Vantage instruments, Becton Dickinson. Monoclonal antibody (mAb) DX1 was a gift of Dr. L. Lanier. Antibodies anti-CD4, anti-CD8, anti-panTCR, were purchased from Becton Dickinson. Antibodies anti-Vα24, anti-CD8β, anti-CD56, anti-CD16, anti-p58KIR (NK workshop mAbs GL183 and EB6) were from Immunotech. Anti-CD69 and anti-CD94 were from Pharmingen.

CDR3 TCR sequencing. Total $CD4^-CD8^-$ Vα24JαQ CDR3 sequences were amplified by RT-PCR using Vα24 and constant region specific primers as described previously (Porcelli *et al.*, *Journal of Experimental Medicine 178*:1–16 (1993)), and cloned using a Stratagene pCR-Script™ kit. TCR transcripts from individual T cell clones were amplified by RT-PCR. Sequences for plasmid and PCR DNA products were determined directly on an ABI373A Automated DNA Sequencer.

Cell culture and cytokine assay. Single cell sorts were grown on allogeneic feeders at 50,000/well and 721.221 cells at 5000/well, irradiated (5000 rads), supplemented with 1 μg/mL PHA-P, IL-2 and IL-7 each at 10 U/mL, and propagated as described (Fukaura *et al.*, *Journal of Clinical Investigation 98*:70–77 (1996)). Clones positive for Vα24 and NKR-P1A by flow cytometry and a Vα24JαQ CDR3 TCR sequence were assayed for cytokine secretion. Cells were stimulated (25,000/well) with plate-bound anti-CD3 (1 μg/mL, Immunotech) or control isotype antibody (Sigma) for 4, 8, or 24 hours. Supernatants were collected and assayed for IL-4 and IFN-γ by capture ELISA. After 24 hours, 1 μCi/well of [^{3}H-]thymidine was added and incorporation measured as described (Fukaura *et al.*, *Journal of Clinical Investigation 98*:70–77 (1996)).

CD1 restriction. Restriction experiments using CD1 isoform (CD1a, CD1c, CD1d, and pSRα-neo vector alone) transfected C1R cells was performed as described (Davodeau *et al.*, *Journal of Immunology 158*:5603–5611 (1997)).

II. Results and Discussion

To determine if there was a relationship between the number of circulating $CD4^-CD8^-$ Vα24JαQ$^+$ T cells and type 1 diabetes, a frequency analysis was performed on a set of type 1 diabetes discordant monozygotic twins and triplets. The number of circulating $CD4^-CD8^-$ Vα24JαQ$^+$ T cells present in diabetes-free twins/triplets was compared with those present in their siblings with disease. The percentage of circulating invariant $CD4^-CD8^-$ Vα24JαQ T cells could be determined by multiplying the frequency of invariant Vα24JαQ sequences present in the total $CD4^-CD8^-$ Vα24$^+$ population times the percent of $CD4^-CD8^-$ Vα24$^+$ T cells as measured by flow cytometric analysis (Table 1). No $CD4^-CD8^-$ Vα24JαQ$^+$ T cells were detected in three diabetics despite at least three sorting attempts for each subject. The

percentage of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells in a previously disease-free diabetic twin (patient 6A, Table 1) studied the week of IDDM diagnosis was similar to their long-term IDDM twin and the other diabetics. In all sets of family pairings, the IDDM sibling had markedly lower percentages of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells (P=0.015, paired sign test using only the discordant twins/triplets data).

To determine whether human $V\alpha24J\alpha Q^+$ T cells were functionally altered in at-risk for and type 1 diabetics, single $CD4^-$ and $CD8^-$ mononuclear cells expressing the $V\alpha24^+$ TCR were cloned. The initial analysis was carried out on clones generated from the IDDM non-progressing member of a sibling pair, subject 7A (Table 1). All clones expressed the invariant $V\alpha24J\alpha Q$ junctional sequences conserving the germ line encoded amino acids Vα24 (-CVVS:) and JαQ (:DRGST-). Eight of ten clones were $V\beta11^+$ and two were $V\beta13^+$. All of the clones were $CD4^-$, and uniformly negative when stained for the CD8β-chain. Surface expression for $CD8\alpha\alpha^+$ appeared to reflect activation state, as staining for this marker reverted to negative 2–3 weeks post-stimulation. All T cells expressed the human homologue of the murine NK1.1 molecule, NKP-P1A (Lanier *et al.*, *Journal of Immunology 153*:2417–2428 (1994)), and the C-type lectins encoded by the NK locus, CD69 and CD94.

CD1d restriction was assessed by co-cultivating the $V\alpha24J\alpha Q^+$ T-cell clones with C1R cells transfected with a CD1d or control expression vector (Exley, *et al.*, *J. Exp. Med. 186*1-11 (1997)). A T-cell clone (4.2) with a non-invariant TCR α-chain (Vα24N3Jα6) was included as a negative control. All T-cell clones except 3.5, 3.8, and the control clone 4.2 specifically proliferated in response to the CD1d transfectant. All of the clones except 3.5 and 4.2 secreted IL-4 and IFN-γ in a CD1d specific manner . Clone 3.5 secreted only IFN-γ in response to CD1d. The fine specificity of the clones for CD1d was tested by using C1R targets transfected with either CD1a, CD1c, CD1d, or vector alone. Only CD1d-expressing target cells specifically stimulated each of the $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ clones as assessed by IL-4 and IFN-γ secretion.

A panel of $V\alpha24J\alpha Q^+$ T-cell clones was raised from: 1) the twins/triplets discordant for type 1 diabetes (*see* Table 1); 2) from an additional four at-risk non-progressors with elevated serum IL-4 levels; and 3) two haplotype (DR3/DR2 and DR4/DRX) matched normal controls.

Twenty-five out of 28 clones raised from the at-risk non-progressors among the discordant twins/triplets secreted both IL-4 and IFN-γ (>10 pg/ml) on stimulation with anti-CD3. The other three clones produced only IFN-γ. Unlike the other non-progressing twins, only one clone from the triplet 1A secreted modest amounts of IL-4 when stimulated. Only a single attempt to generate clones from this subject was made due to their subsequent entry into a clinical trial. All of the 56 clones raised from the diabetic twins/triplets secreted only IFN-γ with anti-CD3 stimulation, and diabetic twins 4B and 5B had no identifiable $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells (Table 1). There was no difference in the proliferative response to anti-CD3 between the clones raised from diabetics or other subjects. The new onset type 1 twin 6A (Table 1) had 9/9 $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T-cell clones that secreted only IFN-γ. This suggests that the Th1 phenotype seen in the new onset twin was not related to duration of diabetes but occurred prior to, or concurrent with, the onset of overt disease.

An additional set of 33 clones were generated from four at-risk non-progressors and 18 clones raised from MHC haplotype matched controls. Clones raised from these subjects were phenotypically similar to the diabetes-free twins and a series of invariant $V\alpha24J\alpha Q^+$ T-cell clones previously described (Exley *et al.*, *J. Exp.Med. 186*:1–11 (1997); Davodeau *et al.*, *J. Immunol. 158*:5603–5611 (1997)). Thus, all $V\alpha24J\alpha Q$ T-cell clones raised from type 1 patients showed an extreme Th1 bias, making them incapable of providing the IL-4 necessary for the initiation of Th2 responses. In fact, unopposed IFN-γ secretion should promote a strong cellular immune response, and could augment or initiate a Th1-dominated cellular attack on β cells (von Herrath *et al.*, *J. Exp. Med. 185*:531–539 (1997); Denkers *et al.*, *J. Exp. Med. 184*:131–139 (1996)).

The functional studies on the $V\alpha24J\alpha Q^+$ T cells from the discordant twins/triplets, at-risk non-progressors, and controls suggested that these two groups had polarized cell mediated immune responses. To further assess this possibility, serum IL-4 and IFN-γ were determined in 14 at-risk IDDM non-progressors who had remained IDDM-free despite a 50% risk of having developed diabetes during the period of study (Verge *et al.*, *Diabetes 45*:926–933 (1996)). This cohort was defined by having remained healthy despite five or more years follow-up after diagnosis of type 1 diabetes in a first degree relative and being islet cell antibody+ (ICA+) with any two of the following autoantibodies: anti-GAD, anti-IA2, or insulin autoantibodies. Seven

of 14 type 1 non-progressors had markedly elevated levels of serum IL-4, six of whom also had elevated IFN-γ (0.2–35 ng/ml). Despite the elevation of cytokines in the serum from 7 of 14 non-progressors, all seven IL-4$^+$ individuals have remained healthy with no evidence of chronic infectious, or atopic/allergic illnesses. The remaining 7 had levels for both cytokines below the detection limit of the ELISA (0.015 ng/ml). Five of 14 individuals in this group were found to have the strongly protective MHC allele DQB1*0602 and therefore are not at the same risk of progression as the remaining nine members of this cohort (Pugliese *et al.*, *Diabetes 44*:608–613 (1995)). Three of these five individuals had elevated serum IL-4 and IFN-γ levels. This was in contrast to the finding that IL-4 could not be detected in the serum (before or after diagnosis of type 1 diabetes) in 12 individuals with identical autoantibody status who developed IDDM after five or more years of follow-up.

Elevated cytokines were also detected in archival serum samples obtained from 3/23 individuals at the time of diagnosis of type 1 diabetes and in 5/26 type 2 diabetics who did not have autoantibodies or a family history of type 1 diabetes. When compared to either normals, antibody positive first degree relatives, recent onset diabetics, long-term diabetics (IDDM >2 years), autoantibody negative first degree relatives, or untreated multiple sclerosis patients (MS), the frequency of serum IL-4$^+$ individuals was significantly elevated in the non-progressor cohort. The authenticity of the detected IL-4 was independently confirmed by using another set of ELISA antibodies, binding to soluble recombinant IL-4 receptor produced in insect cells, and by Western blot.

The results demonstrate a relationship between elevated serum IL-4 levels and resistance to progression of an autoimmune disorder. Prolonged hyperglycemia as an explanation for the absence of IL-4 in type 1 diabetics seems less likely since IL-4 was detected in the serum of type 2 diabetics. The presence of elevated IL-4 levels was not an absolute predictor of IDDM resistance since only half of the resistant cohort had elevated serum IL-4 levels, as did 3/23 diabetics on or about the time of diagnosis.

In the NOD mouse, compelling evidence exists that IL-4 exerts a dominant negative effect on progression to IDDM (Rapoport *et al.*, *J. Exp. Med. 178*:87–99 (1993); Mueller *et al.*, *J. Exp. Med. 184*:1093–1099 (1996); Fox *et al.*, *J. Immunol. 158*:2414–2424 (1997)).

Differentiation of T cells into IL-4 secreting Th2 effector cells requires IL-4 priming (Abbas *et al.*, *Nature 383*:787–793 (1996)). While this proposed function for NK1.1$^+$ T cells was not obligatory for all Th2 immune responses (Bendelac *et al.*, *Ann. Rev. Immunol. 15*:535–562 (1997); Brown *et al.*, *J. Exp. Med. 184*:1295–1304 (1996); Smiley *et al.*, *Science 275*:977–979 (1997)), T cell IL-4 secretion was noted to be markedly diminished in a CD1 knockout background (Smiley *et al.*, *Science 275*:977–979 (1997); Chen *et al.*, *Immunity 6*:459–467 (1997); Mendiratta *et al.*, *Immunity 6*:469–477 (1997)). NK1.1$^+$ T cells were present in diminished numbers and decreased in frequency prior to the onset of disease in several murine models of autoimmunity (Bendelac *et al.*, *Ann. Rev. Immunol. 15*:535–562 (1997); Vicari *et al.*, *Immunology Today 17*:71–76 (1996); Takeda *et al.*, *J. Exp. Med. 177*:155–164 (1993); Mieza *et al.*, *J. Immunol. 156*:4035–4040 (1996); Gombert *et al.*, *Int'l Immunol. 8*:1751–1758 (1996)). In these models, autoimmunity was temporally accelerated by depletion of NK1.1$^+$ T cells and delayed by generating mice transgenic for the Vα14Jα281 TCR. Diabetes was also prevented in the NOD mouse by adoptive transfer of a population harboring the NK1.1-like class of T cell (Baxter *et al.*, *Diabetes 46*:572–582 (1997)).

In summary, type 1 diabetes was associated with an extreme Th1 phenotype for Vα24JαQ$^+$ T cells and a decrease in their circulating frequency. The data presented herein provide a strong link between Vα24JαQ$^+$ T cells and human type 1 diabetes, suggesting that they are functionally related to the resistance or progression of this autoimmune disease in humans.

TABLE 1

Twins/Triplets	DN#%	%DNVα24$^+$ in total lymphocytes	Vα24JαQ DN sequence frequency	Vα24JαQ%
1A/IL-4$^+$	0.74	0.04	20/22	0.036
1B/IDDM	0.95	0.01	10/19	0.005
1C/IDDM	0.76	0.04	9/22	0.016
2A	2.1	0.37	9/10	0.33
2B/IDDM	3.1	0.025	31/31	0.025
3A	1.1	0.04	8/12	0.027

Twins/Triplets	DN#%	%DNVα24+ in total lymphocytes	Vα24JαQ DN sequence frequency	Vα24JαQ%
3B/IDDM	1/89	0.01	5/15	0.003
4A	1.21	0.02	4/13	0.006
4B/IDDM	0.31	0.006	0*	0
5A	0.58	0.06	8/12	0.04
5B/IDDM	0.98	0	0*	0
6A/new IDDM	0.89	0.03	7/26	0.008
6B/IDDM	2.62	0.03	8/23	0.01
brother/sister 7A/IL-4+	2.54	0.03	8/12	0.017
7B/IDDM	1.08	0.005	0/18	0

The frequency of Vα24JαQ TCR sequences was determined by sorting all $CD4^-CD8^-$ $\alpha\beta TCR^+$ T cells, amplifying all Vα24 transcripts and sequencing the TCR CDR3 region; the percentage of cells that were invariant $CD4^-CD8^-$ Vα24JαQ in total mononuclear cells was calculated by multiplying the sequence frequency by the $CD4^-CD8^-V\alpha24^+$% of total mononuclear cells determined by flow cytometry. #DN=$CD4^-CD8^-$. *No Vα24 PCR products were detected in three attempts. $IL\text{-}4^+$ indicates subject with high serum IL-4.

All references cited are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the art that the invention may be performed within a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

What is Claimed is:

1. A method for determining the likelihood that a human subject has, or will develop, an autoimmune disease, said method comprising:
   a) determining the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells in said human subject;
   b) comparing the number determined in step a) with the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells in a control group made up of individuals that do not have an autoimmune disease; and
   c) correlating the likelihood of said human subject having or developing said autoimmune disease based upon the comparison of step b), wherein said likelihood increases as the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells in said subject decreases relative to the number of such cells in said control group.

2. The method of claim 1, wherein said autoimmune disease is selected from the group consisting of: multiple sclerosis; systemic lupus erythematosus; rheumatoid arthritis; type 1 diabetes; myasthenia gravis; psoriasis; scleroderma; Sjögen's disease; and idiopathic thrombocytopenia purpura.

3. The method of claim 2, wherein said autoimmune disease is type 1 diabetes.

4. A method for determining the likelihood that a human subject has or is likely to develop an autoimmune disease, said method comprising:
   a) determining the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ in said human subject;
   b) comparing the percentage determined in step a) with the percentage of total T cells that are $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ in a control group made up of individuals that do not have an autoimmune disease; and
   c) correlating the likelihood of said subject having or developing said autoimmune disease based upon the comparison of step b), wherein said likelihood increases as the percentage of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ cells in said subject decreases relative to the percentage of such cells in said control group.

5. The method of claim 4, wherein the percentage of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ in said subject and said control group is determined by:
   a) sorting $CD4^-CD8^-$ $\alpha\beta TCR^+$ T cells by flow cytometry;
   b) amplifying $V\alpha24$ transcripts and sequencing the CDR3 T cell receptor region to determine frequency; and
   c) multiplying said frequency by the percentage of cells that are $CD4^-CD8^-$ $V\alpha24^+$.

6. The method of claim 4, wherein said autoimmune disease is selected from the group consisting of: multiple sclerosis; systemic lupus erythematosus; rheumatoid arthritis; type 1 diabetes; myasthenia gravis; psoriasis; scleroderma; Sjögen's disease; and idiopathic thrombocytopenia purpura.

7. The method of claim 6, wherein said autoimmune disease is type 1 diabetes.

8. A method for determining the likelihood that a human subject has or will develop an autoimmune disease comprising:
   a) isolating $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells from said human subject;
   b) determining the extent to which said cells secrete IL-4; and
   c) correlating the secretion of IL-4 determined in step b) with the likelihood of said human subjects developing said autoimmune disease, wherein said likelihood decreases as said secretion increases.

9. The method of claim 8, wherein said cells in step b) are stimulated with a T cell activating factor.

10. The method of claim 9, wherein said T cell activating factor is anti-CD3 antibody.

11. The method of claim 8, wherein said autoimmune disease is selected from the group consisting of: multiple sclerosis; systemic lupus erythematosus; rheumatoid arthritis; type 1 diabetes; myasthenia gravis; psoriasis; scleroderma; Sjögen's disease; and idiopathic thrombocytopenia purpura.

12. The method of claim 11, wherein said autoimmune disease is type 1 diabetes.

13. A method of treating a human subject that has or is likely to develop an autoimmune disease, comprising increasing the number of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells in said human subject.

14. The method of claim 13, wherein said increase is accomplished by clonal expansion of said human subject's own $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells.

15. The method of claim 14, wherein cells are clonally expanded by exposing said cells to CD1d.

16. The method of claim 14, wherein said cells are removed from said subject prior to clonal expansion.

17. The method of claim 15, wherein said CD1d is administered to said subject *in vivo*.

18. The method of claim 13, wherein said autoimmune disease is selected from the group consisting of: multiple sclerosis; systemic lupus erythematosus; rheumatoid arthritis; type 1 diabetes; myasthenia gravis; psoriasis; scleroderma; Sjögen's disease; and idiopathic thrombocytopenia purpura.

19. The method of claim 18, wherein said autoimmune disease is type 1 diabetes.

INTERNATIONAL SEARCH REPORT

Internat. .al Application No
PCT/US 98/26868

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/68 G01N33/564

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUMIDA, T. ET AL.: "Selective Reduction of T Cells Bearing Invariant Valpha24JalphaQ Antigen Receptor in Patients with Systemic Sclerosis" THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 4, 1 October 1995, pages 1163-1168, XP002104249	1-7
Y	see the whole document	8-19

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 1999	11/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/26868

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	BENDELAC, A. ET AL.: "Mouse CD1-Specific NK1 T Cells: Development, Specificity, and Function" ANNU. REV. IMMUNOL. , vol. 15, 1997, pages 535-562, XP002104250 cited in the application	8-19
A	see abstract see page 535 - page 537, paragraph 3 see page 538, paragraph 2 - page 540, paragraph 2 see page 546 see page 550, paragraph 2 - page 556	1-7
A	PORCELLI, S. ET AL.: "Analysis of T Cell Antigen Receptor (TCR) Expression by Human Peripheral Blood CD4-8-alpha/beta T Cells Demonstrates Preferential Use of Several Vbeta Genes and an Invariant TCR alpha Chain" THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, no. 1, 1 July 1993, pages 1-16, XP002104251 cited in the application see abstract see page 14, column 1, line 1 - column 2, line 5	1-19
A	WO 94 19470 A (HOECHST JAPAN ;WATANABE HIROSHI (JP); YAMAGATA NOBUYUKI (JP); TANI) 1 September 1994 see abstract see page 1, paragraph 2 see page 6, paragraph 2	1-4,6-8, 11-13, 18,19

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 26868

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-19
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat. al Application No
PCT/US 98/26868

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9419470 A	01-09-1994	JP 6298662 A	25-10-1994
		AU 686134 B	05-02-1998
		AU 6042894 A	14-09-1994
		CA 2134083 A	01-09-1994
		EP 0667908 A	23-08-1994
		US 5648332 A	15-07-1997